COMPOUNDS AND METHODS FOR MODULATING ACTIVATION OF NF-κB

TECHNICAL FIELD

The present invention relates generally to compositions and methods for modulating the activation of nuclear factor κB (NF-κB). The invention is more particularly related to agents that modulate ubiquitination of phosphorylated IκBα and/or IκBβ and to methods for treating diseases associated with NF-κB activation. Modulating agents encompassed by the present invention include E3 ubiquitin ligases, and portions and variants thereof.

BACKGROUND OF THE INVENTION

NF-κB is a transcription factor that plays a pivotal role in the highly specific pattern of gene expression observed for immune, inflammatory and acute phase response genes, including interleukin 1, interleukin 8, tumor necrosis factor and certain cell adhesion molecules. Like other members of the Rel family of transcriptional activators, NF-κB is sequestered in an inactive form in the cytoplasm of most cell types. A variety of extracellular stimuli including mitogens, cytokines, antigens, stress inducing agents, UV light and viral proteins initiate a signal transduction pathway that ultimately leads to NF-κB release and activation.

Important modulators of NF-κB activation are the inhibitor proteins IκBα and IκBβ (referred to herein as IκB), which associate with (and thereby inactivate) NF-κB in the cytoplasm of nonstimulated cells. Activation and nuclear translocation of NF-κB occurs following signal-induced phosphorylation of IκB, which leads to proteolysis via the ubiquitin pathway. For IκBα, the stimulus-induced phosphorylation at serines 32 and 36 renders the inhibitor a target for ubiquitination at lysines 21 and 22, resulting in degradation. Similarly, phosphorylation of IκBβ at serines 19 and 23 renders the inhibitor a target for ubiquitination at lysine 9. However, the component(s) of the ubiquitin system mediating IκB recognition have not been identified.

Degradation of a protein via the ubiquitin pathway proceeds by two discrete and successive steps: (a) covalent attachment of multiple ubiquitin molecules to the protein substrate, and (b) degradation of the targeted protein by the 26S proteasome complex. The ubiquitin pathway consists of several components that act in concert and in a hierarchical manner (for reviews, *see* Ciechanover, *Cell 79*:13, 1994; Hochstrasser, *Curr. Op. Cell. Biol. 7*:215, 1995; Jentsch and Schlenker, *Cell 82*:881, 1995; Deshaies, *Trends Cell Biol. 5*:428, 1995). One such component, a single E1 enzyme, carries out activation of ubiquitin. Several major species of E2 enzymes have been characterized in mammalian cells, plants, and yeast. E2 enzymes probably bind to the ligase E3 (Reiss and Hersko, *J. Biol. Chem. 265*:3685, 1990; Dohmen et al., *Proc. Natl. Acad. Sci. USA 88*:7351, 1991) and it appears that each E2 enzyme can act with one or more E3 proteins (Nuber et al., *J. Biol. Chem. 271*:2795, 1996; Orian et al., *J. Biol. Chem. 270*:21707,1995; Stancovski et al., *Mol. Cell. Biol. 15*:7106, 1995; Gonen et al., *J. Biol. Chem. 271*:302,1996).

Only few E3 enzymes (ubiquitin ligases) have been described. Mammalian E3α (UBR1 in yeast) and E3β recognize protein substrates via their free N-terminal amino acid residues ("N-end rule"; Varshavsky, *Cell 69*:725, 1992; Hershko and Ciechanover, *Ann. Rev. Biochem. 61*:761, 1992). Cdc53 is probably an E3 involved in targeting phosphorylated G1 cyclins (Willems et al., *Cell 86*:453, 1996). E6-AP is involved in recognition of p53 (Scheffner et al., *Cell 75*:495, 1993), and a series of unique E6-AP homologous proteins have been identified (Huibregtse et al., *Proc. Natl. Acad. Sci. USA 92*:2563, 1995): Nedd4 is involved the degradation of the epithelial Na^+ channel (Staub et al, *Embo J. 15*:2371, 1996) and RSP5 (NIP1) is involved in tagging the permeases Gap1 and Fur1 (Hein et al., *Mol. Microbiol. 18*:77, 1995), whereas Pub1 targets Cdc25 (Nefsky and Beach, *EMBO J. 15*:1301, 1996). Several other E3 enzymes that have been recently isolated appear to be involved in the degradation of c-Fos, a subset of muscle proteins, and in the processing of p105, the NF-κB precursor (Orian et al., *J. Biol. Chem. 270*:21707, 1995; Stancovski et al., *Mol. Cell. Biol. 15*:7106, 1995; Gonen et al., *J. Biol. Chem. 271*:302,1996). Thus, it appears that the ligases represent a large, mostly unraveled family of enzymes and, except for the mode of recognition of

the "N-end rule" ligases (E3α and E3β), the recognition motifs of all other known substrates of the ubiquitin system have not been identified.

Accordingly, there is a need in the art for an improved understanding of IκB degradation via the ubiquitin pathway, and for the identification of modulators of this degradation process for use in treating diseases associated with activation of NF-κB. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods for modulating the activation of nuclear factor κB (NF-κB) by modulating ubiquitination of phosphorylated IκBα and/or IκBβ. Within one aspect, the present invention provides isolated human E3 ubiquitin ligase polypeptides. Such polypeptides may comprise a human E3 ubiquitin ligase sequence as recited in SEQ ID NO:16, or a portion or variant thereof that differs in one or more amino acid substitutions, insertions, deletions and/or additions, such that the polypeptide (a) enhances ubiquitination of phosphorylated IκB or (b) binds to phosphorylated IκB and inhibits ubiquitination of phosphorylated IκB. Within certain embodiments, such a polypeptide may have the sequence recited in SEQ ID NO:16 or a variant thereof that differs in one or more amino acid deletions, insertions or substitutions at no more than 20% of the amino acid residues in SEQ ID NO:16, such that the polypeptide enhances ubiquitination of phosphorylated IκB. Within further embodiments, such a polypeptide may comprise a portion of a human E3 ubiquitin ligase, or variant of such a portion, wherein the portion binds to phosphorylated IκB and inhibits ubiquitination of phosphorylated IκB.

The present invention further provides, within other aspects, isolated polynucleotides that encode a polypeptide as described above. Within certain embodiments, such polynucleotides may encode a portion of a human E3 ubiquitin ligase, or variant of such a portion, as described above. Antisense polynucleotides comprising at least 10 consecutive nucleotides complementary to such a polynucleotide

are also provided. Expression vectors comprising such a polynucleotide, and host cells transformed or transfected with such an expression vector, are further provided.

Within further aspects, the present invention provides pharmaceutical compositions comprising a polypeptide or polynucleotide as described above in combination with a physiologically acceptable carrier.

Within other aspects, the present invention provides isolated antibodies, and antigen binding fragments thereof, that bind to a human E3 ubiquitin ligase having a sequence recited in SEQ ID NO:16. Such antibodies may be monoclonal.

Within further aspects, pharmaceutical compositions are provided, comprising an antibody or fragment thereof as described above in combination with a physiologically acceptable carrier.

The present invention further provides methods for modulating NF-κB activity in a patient, comprising administering to a patient a pharmaceutical composition as described above.

Within further aspects, the present invention provides methods for treating a patient afflicted with a disorder associated with NF-κB activation, comprising administering to a patient a therapeutically effective amount of a pharmaceutical composition as described above, and thereby treating a disorder associated with NF-κB activation. Such disorders include inflammatory diseases, autoimmune diseases, cancer and viral infection.

Within further aspects, the present invention provides methods for screening for an agent that modulates NF-κB activity, comprising the steps of: (a) contacting a candidate agent with a human E3 ubiquitin ligase polypeptide, wherein the polypeptide comprises a sequence recited in SEQ ID NO:16 or a portion or variant thereof that differs in one or more amino acid substitutions, insertions, deletions or additions, such that the polypeptide enhances ubiquitination of phosphorylated IκB, under conditions and for a time sufficient to permit interaction between the polypeptide and candidate agent; and (b) subsequently evaluating the ability of the polypeptide to enhance ubiquitination of phosphorylated IκB, relative to a predetermined ability of the polypeptide to enhance ubiquitination of phosphorylated IκB in the absence of

candidate agent; and therefrom identifying an agent that modulates NF-κB activity. Candidate agents for use within such screens include, but are not limited to, small molecules present within a combinatorial library.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D are autoradiograms depicting the results of SDS-PAGE analysis of ubiquitination assays performed in the presence and absence of various IκB E3 recognition motifs. Unless otherwise indicated, the substrate was an ^{35}S-labelled, HA-tagged IκB polypeptide that was phosphorylated and NF-κB complex-associated.

In Figure 1A, lane 1 shows the ubiquitination of an IκBα polypeptide that contains alanine residues at positions 32 and 36 (S32/36A; SEQ ID NO:13) and lane 2 shows the ubiquitination of a non-phosphorylated wild-type IκBα polypeptide (SEQ ID NO:12). In lanes 3-14, the ubiquitination substrate was wild-type IκBα (SEQ ID NO:12). In lane 3, ubiquitination was performed in the absence of ATP; and in lanes 4-14 the reaction was performed in the presence of ATPγS with (lanes 5-14) or without (lane 4) an IκB E3 recognition motif or other peptide. The peptides shown are: 400μM c-Fos phosphopeptide (ppFos (SEQ ID NO:10), lane 5); 400μM serine 32, 36 to alanine substituted IκBα peptide (pp21S/A (SEQ ID NO:11), lane 6); 40μM doubly phosphorylated IκBα peptide (pp21 (SEQ ID NO:9), lane 7); 400μM non-phosphorylated IκBα peptide (p21 (SEQ ID NO:9), lane 8); 100μM singly phosphorylated IκBα peptides (ppS32 (SEQ ID NO:9), lane 9; ppS36 (SEQ ID NO:9), lane 10); and 40μM shorter, doubly phosphorylated IκBα peptides (pp19 (SEQ ID NO:8), lane 11); pp15 (SEQ ID NO:7), lane 12; pp11 (SEQ ID NO:6), lane 13; pp7 (SEQ ID NO:5), lane 14).

In Figure 1B, the ubiquitination substrate was free wild type IκBα (SEQ ID NO:12, lanes 1-3) or free S32/36A substituted IκBα (SEQ ID NO:13, lanes 4-6).

The reaction was performed in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5 and 6) of ATPγS. 40μM doubly phosphorylated IκBα peptide (pp21 (SEQ ID NO:9) was added to the conjugation reaction mixture in the samples shown in lanes 3 and 6.

In Figure 1C, the ubiquitination of bulk cellular proteins in HeLa extract is shown. Lane 1 shows the ubiquitination in the absence of ATP, and lane 5 shows the ubiquitination in the presence of ATP. In lanes 3-5, an IκB E3 recognition motif or other peptide was added: 40μM doubly phosphorylated IκBα peptide (pp21 (SEQ ID NO:9), lane 2); 400μM c-Fos phosphopeptide (ppFos (SEQ ID NO:10), lane 3); and 400μM non-phosphorylated IκBα peptide (p21 (SEQ ID NO:9), lane 4).

In Figure 1D, the ubiquitination substrate was phosphorylated (lanes 2-7) or non-phosphorylated (lane 1) wild type IκBβ (SEQ ID NO:14). Reactions were performed in the absence (lane 2) or presence (lanes 1, 3-7) of ATPγS, and with (lanes 4-7) or without (lanes 1-3) an IκB E3 recognition motif or other peptide. The peptides shown are: 40μM doubly phosphorylated IκBα peptide (pp21 (SEQ ID NO:9), lane 4); 400μM c-Fos phosphopeptide (ppFos (SEQ ID NO:10), lane 5); 40μM doubly phosphorylated IκBα peptide (pp19 (SEQ ID NO:8), lane 6); and 400μM non-phosphorylated IκBα peptide (p21 (SEQ ID NO:9), lane 7).

Figure 2 is an autoradiogram depicting the results of an *in vitro* ubiquitin-dependent degradation assay performed using extracts from stimulated HeLa cells. In each lane of the SDS-PAGE, the level of phosphorylated (upper band) and non-phosphorylated (lower band) HA-tagged IκBα polypeptide (SEQ ID NO:12) following the degradation assay is shown. Lane 1 shows the level of these polypeptides following a degradation assay performed without ATP. In lanes 2-6, ATP was included in the reaction mixture. 40μM candidate modulating agents were added to the reactions shown in lanes 3-6: doubly phosphorylated IκBα peptide (pp21 (SEQ ID NO:9), lane 3); doubly phosphorylated IκBα peptide (pp19 (SEQ ID NO:8), lane 4); c-Fos phosphopeptide (ppFos (SEQ ID NO:10), lane 5); and non-phosphorylated IκBα peptide (p21 (SEQ ID NO:9), lane 6).

Figure 3A is an autoradiogram depicting the results of SDS-PAGE analysis of ubiquitination assays performed using flow-through fractions of HeLa cell

lysate fractionated over modulating agent columns. In each case, the substrate was a ^{35}S-labelled, HA-tagged IκBα polypeptide (SEQ ID NO:12) that was phosphorylated and NF-κB complex-associated. Lane 1 shows the level of ubiquitination using a non-fractionated extract. In lanes 2-9, the extract was fractionated over a peptide-Sepharose® column. The peptides used were: c-Fos phosphopeptide (ppFos (SEQ ID NO:10), lane 2); serine 32, 36 to alanine substituted IκBα peptide (pp21S/A (SEQ ID NO:11), lane 3); doubly phosphorylated IκBα peptide (pp21 (SEQ ID NO:9), lanes 4-6); and doubly phosphorylated IκBα peptide (pp19 (SEQ ID NO:8), lanes 7-9). In addition, reticulocyte Fraction II (160 μg) was added to the ubiquitination reactions shown in lanes 5 and 8, and Fraction I (160 μg) was added to the reactions in lanes 6 and 9.

Figure 3B is an autoradiogram showing the ubiquitination of bulk cellular proteins in HeLa extract. Lane 1 shows the ubiquitination in the absence of ATP, and lane 2 shows the ubiquitination in the presence of ATP, but without candidate modulating agent. In lanes 3-6, candidate modulating agents were added: 40μM doubly phosphorylated IκBα peptide (pp19 (SEQ ID NO:8), lane 3); 400μM c-Fos phosphopeptide (ppFos (SEQ ID NO:10), lane 4); 400μM serine 32, 36 to alanine substituted IκBα peptide (pp21S/A (SEQ ID NO:11), lane 5); and 40μM doubly phosphorylated IκBα peptide (pp21 (SEQ ID NO:9) , lane 6).

Figures 4A-4F are micrographs showing the effect of candidate modulating agents on nuclear NF-κB translocation. In Figures 4A-C, pp21 (Figures 4A and 4B) or ppFos (Figure 4C) was microinjected into the cytoplasm of HeLa cells. Cells were then activated immediately with TNFα and immunostained with anti-p65 antibodies. In Figures 4D-F, pp21 (Figure 4D) or ppFos (Figure 4F) was injected into the cytoplasm of human vascular endothelial cells (HUVEC). Cells were then activated immediately with TNFα and immunostained with anti-E-selectin antibodies. Figure 4E is a phase contrast photograph of Figure 4D. In each micrograph, the injected cells are marked by large arrows. A non-injected, E-selectin negative cell is marked by a small arrow in Figures 4D and 4E.

Figures 4G and 4H are graphs presenting a summary of the microinjection experiments shown in Figures 4A - 4F. In Figure 4G, the percent of HeLa cells displaying nuclear p65 staining is shown. 90 and 42 cells were microinjected with pp21 and ppFos, respectively. Figure 4H shows the percent of HUVEC displaying E-selectin staining. 160 and 36 cells were microinjected with pp21 and ppFos, respectively. For each graph, column 1 shows the level in the absence of an IκB E3 recognition motif or other peptide and TNFα activation. Columns 2-4 show the level following TNFα activation in the absence of peptide (column 2) or in the presence of pp21 (column 3) or ppFos (column 4).

Figure 5 is an autoradiogram depicting the results of a Western blot analysis showing the immunoprecipitation of pIκBα-associated ubiquitin-ligase activity from TNFα-activated cells. The pIκBα/NF-κB complex was immunoprecipitated from proteasome-inhibited, TNFα-stimulated or non-stimulated HeLa cells and subjected to *in vitro* ubiquitination upon addition of ubiquitin, ATP-γS and the following components: lane 1, UBC5C; lane 2, UBC5C and E1; lane 3, none; lanes 4-6, UBC5C and E1 as indicated; lane 7, UBC5C, E1 and pIκBα-peptide; lane 8, UBC5C, E1 and serine-substituted IκBα peptide; lane 9, a sample of TNFα-stimulated HeLa lysate. Cell-stimulation is indicated in the TNFα row. Monomeric and ubiquitin-conjugated IκBα are marked at the left, bottom and top of the figure.

Figure 6 is an autoradiogram illustrating the association of the ubiquitin-ligase with the IκBα/NF-κB complex, following IKK-phosphorylation of IκBα at the DSGLDS (SEQ ID NOs:8 and 19) site. ^{35}S-labeled IκBα/NF-κB complex immunopurified from non-activated cells was phosphorylated by IKK-2EE (where marked by + at the top), incubated with non-activated HeLa lysate as an E3 source, washed and subjected to *in vitro* ubiquitination in the presence of ATPγS, ubiquitin, E1, UBC5C (except where an excluded component is indicated by Abst Ub-Enz). Lanes 2-7 show phosphorylation by IKK; lanes 1 and 3-7 show the effect of incubation with HeLa lysate; in lane 4, a pIκBα peptide was added during the incubation with HeLa lysate; in lane 5, serine-substituted IκBα peptide was added during HeLa incubation; in

lane 6, E1 was omitted from the ubiquitination stage; and in lane 7, UBC5C was omitted during ubiquitination.

Figures 7A and 7B illustrate the identification of IκBα-binding proteins associated with ubiquitin-ligase activity. Figure 7A is a photograph showing Colloidal Blue staining of SDS-polyacrylamide gel samples of immunopurified fractions containing IκBα/NF-κB and associated proteins. IκBα/NF-κB complex was phosphorylated by IKK-2EE (lanes 2, 3) or mock-phosphorylated and used to adsorb the ubiquitin-ligase from HeLa lysate (lanes 1, 2). Molecular-size markers (κD) are indicated on the right. Proteins identified by mass-spectrometry analysis are indicated on the left. Gel-sites corresponding to the bands associated with the ubiquitin-ligase activity (p54 and p58) are marked on the left by brackets. Figure 7B is an autoradiogram of proteins adsorbed onto pIκBα/NF-κB from ^{35}S-labeled HeLa cells. Radiolabeled HeLa lysate was incubated with IKK-phosphorylated antibody-immobilized IκBα/NF-κB complex. The immune-complexes were then washed, eluted and analyzed by SDS-PAGE and autoradiography. Lane 1 shows non-phosphorylated IκBα/NF-κB complex incubated with HeLa lysate; lanes 2-4 show phosphorylated IκBα/NF-κB-complex incubated with HeLa lysate in the absence (lane 2) or presence of pIκBα-peptide (lane 3) or serine-substituted IκBα-peptide (lane 4). Indicated on the left are molecular size markers (κD), Rel A and IκBα bands; indicated in the right are the four pIκBα-associated bands, three of which were displaced by the pIκBα peptide (arrows).

Figures 8A-8D show the results of a mass-spectrum analysis of ubiquitin-ligase associated p54. Figure 8A shows a nanoelectrospray mass spectrum of the unseparated tryptic peptide mixture from the 54 κD gel band excised from a ligase-positive lane (equivalent to lane 2 in Figure 7B). Peaks marked by arrows were fragmented and identified as peptides derived from β-TrCP. The bar indicates the region enlarged in C. Figures 8B and 8C present a comparison of the nanoelectrospray spectra of the 54 κD band associated with (C) and without (B) ubiquitin-ligase activity. The peptide at m/z 714.38 was selected for sequencing. Figure 8D is a fragmentation spectrum of the peptide identified in Figure 8C. A sequence tag was assembled from a

series of doubly charged fragment ions and searched in the *nrdb* data-base for a matching pattern. Fragment masses calculated for the retrieved β-TrCP sequence AAVNVVDFDDKYIVSASGDR (SEQ ID NO:20) were compared with the complete fragmentation spectrum to confirm the match. Peaks matching expected fragment ions are marked by circles.

Figures 9A and 9B present the sequence of a polynucleotide encoding a human E3 ubiquitin ligase (SEQ ID NO:15).

Figure 10 presents a human E3 ubiquitin ligase protein sequence (SEQ ID NO:16).

Figures 11A-11C are Western blots illustrating binding and ubiquitination specificity of E3 ubiquitin ligase family members. Within these figures, mβ-TrCP indicates mouse β-TrCP, hβ-TrCP indicates human β-TrCP, Δβ-TrCP indicates human β-TrCP with a deletion of the F box region and Slimb indicates the Drosophila Slimb protein. Figure 11A illustrates selective binding to pIκBα. Proteins were immunoprecipitated through a FLAG epitope from transfected 293T cells, incubated with immunopurified IκBα/NF-κB complex, which had been treated (-/+ IKK) as indicated and the bound material was analyzed by Western blotting with the indicated antibodies. The top panel shows specific pIκBα binding; the middle panel shows 10% of the substrate flow-through; the bottom panel is a blot of the immunoprecipitated proteins; and molecular size markers (kD) are indicated on the left. Figure 11B shows that β-TrCP-pIκBα binding is abrogated by a phosphopeptide representing the pIκBα degradation motif[1] (pp10), but not by a related non-phosphorylated peptide (pS/A). Figure 11C illustrates *in vitro* ubiquitination of pIκBα by the E3 family member proteins. Immunopurified FLAG-tagged proteins were incubated with ^{35}S-labeled IκBα/NF-κB complexes, treated (-/+ IKK) as indicated and subject to ubiquitination in the presence of ATPγS, ubiquitin, E1 and UBC5C. The IκBα substrate (composed of full-length and two degradation products), pIκBα-polyubiquitin conjugates and molecular size markers are indicated on the left.

Figures 12A and 12B illustrate inhibition of IκBα degradation and NF-κB activation by overexpression of Δβ-TrCP, a dominant negative molecule. Figure

12A is a graph depicting the results of a κB-dependent luciferase assay in P/I-stimulated Jurkat cells transfected with κB-Luc reporter plasmid and the indicated expression vectors (*i.e.*, from left to right, vector alone, vector encoding human β-TrCP, vector encoding human β-TrCP with a deletion of the F box region and vector encoding Drosophila Slimb protein). NF-κB activity is shown as relative (fold) luciferase activity, the non-stimulated empty FLAG vector being the reference (single-fold). Figure 12B depicts the results of western blot analysis of IκBα of phorbol-ester and Ca^{++} ionophore [P/I]-stimulated and non-stimulated Jurkat cells transfected with an empty FLAG vector or Δβ-TrCP. The post-stimulation interval (min) is indicated.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods useful for modulating the activation of nuclear factor κB (NF-κB) and for treating diseases associated with such activation. In particular, the invention is directed to agents that modulate ubiquitination of phosphorylated IκB (*i.e.*, IκBα and/or IκBβ). Such ubiquitination results in the release and activation of NF-κB.

The present invention is based, in part, on the identification and characterization of a human E3 ubiquitin ligase that recognizes phosphorylated and NF-κB-associated IκB. Polypeptides comprising this E3 ubiquitin ligase, as well as portions and other variants thereof, may be used to modulate NF-κB activity *in vitro* or in a patient. Such polypeptides may also be used, for example, to identify agents (such as small molecules) that may be used to modulate NF-κB activity, and to treat disorders associated with abnormal NF-κB activation.

HUMAN E3 UBIQUITIN LIGASE POLYPEPTIDES AND POLYNUCLEOTIDES

It has been found, within the context of the present invention, that a human E3 ubiquitin ligase that migrates as a 54 kD protein binds to, and enhances ubiquitination of, phosphorylated IκBα (phosphorylated IκBα is also designated herein as pIκBα). The sequence of a polynucleotide encoding a human E3 ubiquitin ligase is provided in Figure 9 and SEQ ID NO:15; and a full length human E3 ubiquitin ligase

protein sequence is provided in Figure 10 and SEQ ID NO:16. Human E3 ubiquitin ligase has also been found, within the context of the present invention, to be a member of a family of F-box/WD proteins that includes β-TrCP (Margottin et al., *Mol. Cell 1*:565-574, 1998) and the Drosophila Slimb protein (*see* Jiang and Struhl, *Nature 391*:493-496, 1998). As described in greater detail below, other members of this family share certain properties of E3, and such proteins and variants thereof may be used within certain methods provided herein for E3.

Human E3 ubiquitin ligase polypeptides encompassed by the present invention include native human E3 ubiquitin ligase (also referred to herein as "E3"), as well as portions and other variants thereof. Variants of E3 may differ in sequence from native E3 due to one or more amino acid substitutions, deletions, additions and/or insertions, as described herein, provided that the variant binds to and enhances ubiquitination of an IκB polypeptide as described herein. Preferably, a variant of E3 contains amino acid substitutions at no more than 20%, preferably no more than 15% and more preferably no more than 10%, of the residues recited in SEQ ID NO:16. Variants further include truncated polypeptides and polypeptides containing additional amino acid sequences that have minimal influence on the activity of the polypeptide. A human E3 ubiquitin ligase polypeptide may be of any length, provided that it retains the recited properties. In other words, such a polypeptide may be an oligopeptide (*i.e.*, consisting of a relatively small number of amino acid residues, such as 8-10 residues, joined by peptide bonds), a full length protein (or variant thereof) or a polypeptide of intermediate size (*e.g.*, 20, 50, 200 or 400 amino acid residues).

Certain variants contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. Amino acid substitutions may generally be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and

amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydropathic nature of the polypeptide.

As noted above, certain E3 polypeptides may contain additional amino acid sequences at the amino and/or carboxy termini. For example, an E3 sequence may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. A polypeptide may also, or alternatively, be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.*, poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

The ability of an E3 polypeptide to bind to phosphorylated IκB may be readily determined using any binding assay known to those of ordinary skill in the art. For example, pIκBα/NF-κB complexes may be incubated with immobilized E3 polypeptide, and the level of IκBα binding evaluated using anti-IκBα antibodies (in, for example, a Western blot). Within such assays, an E3 polypeptide should bind detectably to the IκBα; preferably the E3 polypeptide binds at a level that is not substantially diminished relative to the native human E3. In other words, the ability of a variant to bind detectably to phosphorylated and complexed IκBα may be enhanced or unchanged, relative to the native polypeptide, or may be diminished by less than 50%, and preferably less than 20%, relative to the native polypeptide. It will be apparent that other suitable substrates may be substituted for pIκBα/NF-κB complexes within such assays.

The ability of an E3 polypeptide to enhance ubiquitination of phosphorylated IκB may be assessed by incubating the polypeptide with IκBα/NF-κB complex, along with ATPγS, ubiquitin E1 and ubiquitin E2, and detecting the slow-moving IκBα-ubiquitin conjugates by Western blot using IκBα-specific antibodies, as described herein. In general, an E3 polypeptide should result in a detectable level of ubiquitination within such an assay; preferably the level of ubiquitination is not substantially diminished relative to the level of ubiquitination generated by a similar amount of native human E3.

Also encompassed by the present invention are polypeptides comprising a portion or other variant of E3 that retains the ability to bind to phosphorylated IκB, but does not retain the ability to enhance ubiquitination of IκB. Such polypeptides may be readily identified using the binding assays and ubiquitination assays provided herein, and may generally be used to inhibit ubiquitination of IκB. Such polypeptides include those from which the F-box region (*i.e.*, a region of the protein that interacts with one or more components of the ubiquitin cascade) has been deleted. F box regions may generally be identified functionally (*i.e.*, deletion of an F-box region results in a protein that tails to recruit appropriate components of the ubiquitin machinery) and based on the present of an F-box region consensus sequence (*see* Patton et al., Trends in Genet. 14:236-243, 1998). Certain such polypeptides contain a deletion of amino acids 122-168 of SEQ ID NO:16. Within certain embodiments, portions of E3 may comprise 10 to 374 consecutive amino acid residues, preferably 50 to 250, consecutive amino acid residues of the sequence recited in SEQ ID NO:16.

The present invention further provides polynucleotides that encode an E3 polypeptide as provided herein. Any polynucleotide that encodes such a polypeptide, or a portion or variant thereof as described herein, is encompassed by the present invention. Such polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Native DNA sequences encoding a human E3, or portion thereof, may be isolated using any of a variety of hybridization or amplification techniques, which are well known to those of ordinary skill in the art. Within such techniques, probes or primers may be designed based on the E3 sequence provided herein, and may be purchased or synthesized. Libraries from any suitable tissue may be screened. An amplified portion or partial cDNA molecule may then be used to isolate a full length gene from a genomic DNA library or from a cDNA library, using well known techniques. Alternatively, a full length gene can be constructed from multiple PCR fragments. Partial and full length polynucleotides comprising such sequences, other portions of full length polynucleotides, and sequences complementary to all or a portion of such full length molecules, are specifically encompassed by the present invention. In addition, homologues from other species are specifically contemplated, and may generally be prepared as described herein.

Polynucleotide variants of the recited sequences may differ from a native E3 polynucleotide in one or more substitutions, deletions, insertions and/or additions. Preferred variants contain nucleotide substitutions, deletions, insertions and/or additions at no more than 20%, preferably at no more than 10%, of the nucleotide positions. Certain variants are substantially homologous to a native gene, or a portion or complement thereof. Such polynucleotide variants are capable of hybridizing under moderately stringent conditions to a naturally occurring DNA sequence encoding an E3 protein (or a complementary sequence). Suitable moderately stringent conditions include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS). Such hybridizing DNA sequences are also within the scope of this invention.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides

that vary due to differences in codon usage are specifically contemplated by the present invention.

As noted above, the present invention further provides antisense polynucleotides and portions of any of the above sequences. Such polynucleotides may generally be prepared by any method known in the art including, for example, solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* or *in vivo* transcription of DNA sequences that are incorporated into a vector downstream of a suitable RNA polymerase promoter (such as T3, T7 or SP6). Certain portions of a polynucleotide may be used to prepare an encoded polypeptide, as described herein. In addition, or alternatively, a portion may function as a probe (*e.g.*, to detect E3 expression in a sample), and may be labeled by a variety of reporter groups, such as radionuclides, fluorescent dyes and enzymes. Such portions are preferably at least 10 nucleotides in length, and more preferably at least 20 nucleotides in length. Within certain preferred embodiments, a portion for use as a probe comprises a sequence that is unique to an E3 gene. A portion of a sequence complementary to a coding sequence (*i.e.*, an antisense polynucleotide) may also be used as a probe or to modulate gene expression. DNA constructs that can be transcribed into antisense RNA may also be introduced into cells or tissues to facilitate the production of antisense RNA.

Any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Polynucleotides as described herein may be joined to a variety of other nucleotide sequences using established recombinant DNA techniques. For example, a polynucleotide may be cloned into any of a variety of cloning vectors, including plasmids, phagemids, lambda phage derivatives and cosmids. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors and

sequencing vectors. In general, a vector will contain an origin of replication functional in at least one organism, convenient restriction endonuclease sites and one or more selectable markers. Additional initial, terminal and/or intervening DNA sequences that, for example, facilitate construction of readily expressed vectors may also be present. Suitable vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art. Other elements that may be present in a vector will depend upon the desired use, and will be apparent to those of ordinary skill in the art.

Vectors as described herein may generally be transfected into a suitable host cell, such as a mammalian cell, by methods well-known in the art. Such methods include calcium phosphate precipitation, electroporation and microinjection.

E3 polypeptides may generally be prepared using standard automated synthesis techniques or by expression of recombinant DNA encoding the desired polypeptide. In general, peptides may be prepared synthetically using standard techniques, incorporating amino acids and/or amino acid analogs. During synthesis, active groups of amino acids and/or amino acid analogs may be protected as necessary using, for example, a t-butyldicarbonate (t-BOC) group or a fluorenylmethoxy carbonyl (FMOC) group. Amino acids and amino acid analogs may be purchased commercially (*e.g.*, Sigma Chemical Co.; Advanced Chemtec) or synthesized using methods known in the art. Peptides may be synthesized using a solid phase method, in which the peptides are attached to a resin such as 4-methylbenzhydrylamine (MBHA), 4-(oxymethyl)-phenylacetamido methyl- and 4-(hydroxymethyl)phenoxy methyl-copoly(styrene-1% divinylbenzene) (Wang resin), all of which are commercially available, or to p-nitrobenzophenone oxime polymer (oxime resin) which can be synthesized as described by De Grado and Kaiser, *J. Org. Chem. 47*:3258, 1982. Those skilled in the art will realize that the choice of amino acids and/or amino acid analogs will depend, in part, on the specific physical, chemical or biological characteristics desired. Such characteristics are determined, in part, by the method of administration and the target location within a patient.

Selective modification of the reactive groups in a peptide can also impart desirable characteristics. Peptides can be manipulated while still attached to the resin to obtain N-terminal modified compounds such as an acetylated peptide or can be removed from the resin using hydrogen fluoride or an equivalent cleaving agent and then modified. Compounds synthesized containing the C-terminal carboxy group (Wang resin) can be modified after cleavage from the resin or, in some cases, prior to solution phase synthesis. Methods for modifying the N-terminus or C-terminus of a peptide are well known in the art and include, for example, methods for acetylation of the N-terminus or amidation of the C-terminus. Similarly, methods for modifying side chains of the amino acids or amino acid analogs are well known to those skilled in the art of peptide synthesis. The choice of modifications made to reactive groups present on the peptide will be determined by the desired characteristics.

An E3 polypeptide may also be a cyclic peptide. A cyclic peptide can be obtained by inducing the formation of a covalent bond between, for example, the amino group at the N-terminus of the peptide and the carboxyl group at the C-terminus. Alternatively, a cyclic peptide can be obtained by forming a covalent bond between a terminal reactive group and a reactive amino acid side chain or between two reactive side chains. It will be apparent to those of skill in the art that a cyclic peptide is selected based on the desired properties. For example, a cyclic peptide may provide increased stability, increased solubility, decreased immunogenicity or decreased clearance *in vivo*.

A newly synthesized peptide can be purified using a method such as reverse phase high performance liquid chromatography (RP-HPLC) or other methods of separation based on size or charge. Furthermore, a purified peptide can be characterized using these and other well known methods such as amino acid analysis and mass spectrometry.

Alternatively, polypeptides may generally be prepared from nucleic acid encoding the desired polypeptide using well known techniques. To prepare an endogenous protein, an isolated cDNA may be used. To prepare a variant polypeptide, standard mutagenesis techniques, such as oligonucleotide-directed site-specific

mutagenesis may be used, and sections of the DNA sequence may be removed to permit preparation of truncated polypeptides.

In general, any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant polypeptides of this invention. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a DNA sequence that encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast, baculovirus-infected insect cells and animal cells. Following expression, supernatants from host/vector systems which secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. One or more reverse phase HPLC steps can be employed to further purify a recombinant polypeptide.

In general, polypeptides and polynucleotides as described herein are isolated. An "isolated" polypeptide or polynucleotide is one that is removed from its original environment. For example, a naturally-occurring protein is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, polypeptides provided herein are isolated to a purity of at least 80% by weight, more preferably to a purity of at least 95% by weight, and most preferably to a purity of at least 99% by weight. In general, such purification may be achieved using, for example, the standard techniques of ammonium sulfate fractionation, SDS-PAGE electrophoresis, and affinity chromatography. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment.

ANTIBODIES

The present invention further provides antibodies, and antigen-binding fragments thereof, that specifically bind to an E3 polypeptide. As used herein, an antibody, or antigen-binding fragment, is said to "specifically bind" to a polypeptide if it reacts at a detectable level (within, for example, an ELISA) with the polypeptide, and does not react detectably with unrelated proteins. Antibodies may be polyclonal or

monoclonal. Preferred antibodies are those antibodies that inhibit or block E3 activity and within a ubiquitination assay as described herein. Other preferred antibodies (which may be used, for example, in immunokinase assays) are those that immunoprecipitate active E3, as determined using any standard assay, such as an assay provided herein.

Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art (*see, e.g.,* Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988). In one such technique, an immunogen comprising the polypeptide is initially injected into a suitable animal (*e.g.,* mice, rats, rabbits, sheep and goats), preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol. 6*:511-519, 1976, and improvements thereto. Briefly, these methods involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction.

Within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments, which may be prepared using standard techniques. Briefly, immunoglobulins may be purified from rabbit serum by affinity chromatography on Protein A bead columns (Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988) and digested by papain to yield Fab and Fc fragments. The Fab and Fc fragments may be separated by, for example, affinity chromatography on protein A bead columns.

UBIQUITINATION ASSAYS

As noted above, the ability of an E3 polypeptide to modulate ubiquitination of phosphorylated IκB may be assessed by incubating the polypeptide with IκBα/NF-κB complex (or any other suitable substrate), along with ATPγS, ubiquitin E1 and ubiquitin E2, and detecting IκBα-ubiquitin conjugates by, for example, Western blot using IκBα-specific antibodies. IκB polypeptides for use in a ubiquitination assay as described herein may be native human IκBα (SEQ ID NO:1) or IκBβ (SEQ ID NO:3), or may be a variant of a native protein. Polypeptide variants of IκB are generally modified such that the ability of the variant to be phosphorylated and ubiquitinated within a ubiquitination assay as described herein is not substantially diminished. An IκB polypeptide may be labeled. For example, ^{35}S may be incorporated into a IκB polypeptide by *in vitro* translation of the polypeptide in the presence of ^{35}S-methionine, using standard techniques.

An IκB polypeptide may generally be prepared from DNA encoding the polypeptide by expression of the DNA in cultured host cells or by translation using an

in vitro system such as wheat germ extract. If host cells are employed, such cells are preferably are bacteria, yeast, baculovirus-infected insect cells or mammalian cells. The recombinant DNA may be cloned into any expression vector suitable for use within the host cell, using techniques well known to those of ordinary skill in the art. *In vitro* translation of polypeptide may generally be performed according to the manufacturer's instructions.

Expressed IκB polypeptides may be used without purification following *in vitro* translation. Alternatively, a polypeptide may be isolated in substantially pure form. An IκB polypeptide may be isolated to a purity of at least 80% by weight, preferably to a purity of at least 95% by weight, and more preferably to a purity of at least 99% by weight. In general, such purification may be achieved using, for example, the representative purification method described herein or the standard techniques of ammonium sulfate fractionation, SDS-PAGE electrophoresis, and affinity chromatography.

Certain ubiquitination assays may employ a cellular E3 to characterize modulators of E3 activity. Within such assays, cellular extracts from stimulated or non-stimulated Jurkat, HeLa, THP-1 or endothelial cells may be incubated *in vitro* with an IκB polypeptide in the presence of ATP and the phosphatase inhibitor okadaic acid. Cellular extracts may generally be prepared according to the method of Alkalay et al., *Proc. Natl. Acad. Sci. USA 92*:10599, 1995. The incubation is performed under conditions sufficient to result in phosphorylation of the IκB polypeptide (at serines 32 and 36 for IκBα and variants thereof) and association of the phosphorylated polypeptide (pIκB) with the cellular-derived NF-κB complex. For example, IκB polypeptide may be incubated with HeLa or Jurkat cell extract, ATP and okadaic acid. Incubation for 90 minutes at 30°C is generally sufficient to allow phosphorylation of the IκB polypeptide. Following this incubation, the pIκB/NF-κB complex may be immunopurified with, for example, anti-p65 antibodies and subjected to *in vitro* ubiquitination in a cell free system, as described by Alkalay et al., *Proc. Natl. Acad. Sci. USA 92*:10599, 1995. The level of ubiquitination may then be evaluated using the well known techniques of SDS-PAGE, followed by autoradiography.

Under these conditions, a wild type ^{35}S-pIκBα polypeptide generates multiply ubiquitinated species in the presence of ATPγS (*see* Figure 1A, lane 4). Neither ^{35}S-labeled S32/36A mutant of IκBα (lane 1), nor the non-phosphorylated wild type ^{35}S-IκBα (lane 2) are ubiquitinated. However, free forms of either mutant or wild type IκBα are readily conjugated (Figure 1B). Similarly, a free (but not a complex-associated) lysine 21, 22 mutant of IκBα can be ubiquitinated *in vitro*. Thus, unlike ubiquitination assays performed using free IκB polypeptides, the ubiquitination assay provided herein targets only IκB polypeptides that are complex-associated and appropriately phosphorylated.

A ubiquitination assay as described above may be used to identify agents that modulate ubiquitination of IκB. Modulating agents may include antibodies (*e.g.*, monoclonal), peptides, small molecules (*e.g.*, from a combinatorial library) and other drugs that stimulate or, preferably, inhibit ubiquitination of an IκBα and/or IκBβ polypeptide. In general, such agents may be identified by including a candidate modulating agent in the ubiquitination reaction, which may otherwise be performed as described above, and evaluating the effect of the agent on the level of ubiquitination. A suitable concentration of candidate agent for use in such an assay generally ranges from about 0.1 μM to about 1 mM. For peptide candidate agents, a peptidase inhibitor such as Bestatin (40 μg/mL) may also be added, and the amount of peptide preferably ranges from about 10 μM to about 1 mM. A candidate agent that results in a statistically significant effect on the level of ubiquitination is a modulating agent encompassed by the present invention.

Agents may be further evaluated by microinjection of the agent (*e.g.*, about 5 mg/mL of a peptide agent) into a suitable cell (*e.g.*, HeLa cell or primary human vascular endothelial cell). Following microinjection, cells may be stimulated (*e.g.*, with TNFα) and incubated to allow NF-κB activation. In HeLa cells, TNFα induces rapid nuclear translocation of NF-κB into the nucleus, which may be detected by staining with p65-specific antibodies. Modulating agents induce a statistically significant decrease in NF-κB translocation, and may reduce such translocation to undetectable levels.

Primary human vascular endothelial cells (HUVEC) respond to TNFα stimulation by surface expression of NF-κB regulated adhesion proteins such as ICAM-1, V-CAM-1 and E-selectin (Read et al., *Immunity 2*:493,1995; Chen et al., *J. Immunol 155*:3538, 1995). E-selectin expression is particularly NF-κB dependent and is the major inducible endothelial adhesion molecule for initial neutrophil attachment and rolling on activated endothelium. Stimulated cells may be fixed and stained to detect expression of one or more NF-κB regulated adhesion proteins. Microinjection of a polypeptide or other modulating agent results in a statistically significant inhibition of such expression, but does not affect the expression of NF-κB independent adhesion proteins, such as ICAM2.

THERAPEUTIC APPLICATIONS

As noted above, certain E3 polypeptides, polynucleotides, antibodies and other agents as described herein may generally be used as modulating agents to specifically inhibit or enhance cellular NF-κB functions. Modulating agents may also be used to modulate ubiquitination of IκBα and/or IκBβ in a patient, thereby modulating NF-κB cellular function *in vivo*. As used herein, a "patient" may be any mammal, including a human, and may be afflicted with a disease associated with NF-κB activation, or may be free of detectable disease. Accordingly, the treatment may be of an existing disease or may be prophylactic. Diseases associated with NF-κB activation include, but are not limited to, inflammatory diseases, autoimmune diseases, cancer and viral infection.

Treatment refers to administration of a modulating agent as described herein. For administration to a patient, one or more such compounds are generally formulated as a pharmaceutical composition. A pharmaceutical composition may be a sterile aqueous or non-aqueous solution, suspension or emulsion, which additionally comprises a physiologically acceptable carrier (*i.e.*, a non-toxic material that does not interfere with the activity of the active ingredient). Any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of the present invention. Representative carriers include physiological saline solutions,

gelatin, water, alcohols, natural or synthetic oils, saccharide solutions, glycols, injectable organic esters such as ethyl oleate or a combination of such materials. Optionally, a pharmaceutical composition may additionally contain preservatives and/or other additives such as, for example, antimicrobial agents, anti-oxidants, chelating agents and/or inert gases, and/or other active ingredients.

Alternatively, a pharmaceutical composition may comprise a polynucleotide encoding a modulating agent (such that the modulating agent is generated *in situ*) in combination with a physiologically acceptable carrier. In such pharmaceutical compositions, the polynucleotide may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid, bacterial and viral expression systems, as well as colloidal dispersion systems, including liposomes. Appropriate nucleic acid expression systems contain the necessary polynucleotide sequences for expression in the patient (such as a suitable promoter and terminating signal). DNA may also be "naked," as described, for example, in Ulmer et al., *Science 259*:1745-1749, 1993.

Various viral vectors that can be used to introduce a nucleic acid sequence into the targeted patient's cells include, but are not limited to, vaccinia or other pox virus, herpes virus, retrovirus, or adenovirus. Techniques for incorporating DNA into such vectors are well known to those of ordinary skill in the art. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus including, but not limited to, Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A retroviral vector may additionally transfer or incorporate a gene for a selectable marker (to aid in the identification or selection of transduced cells) and/or a gene that encodes the ligand for a receptor on a specific target cell (to render the vector target specific). For example, retroviral vectors can be made target specific by inserting a nucleotide sequence encoding a sugar, a glycolipid, or a protein. Targeting may also be accomplished using an antibody, by methods known to those of ordinary skill in the art.

Viral vectors are typically non-pathogenic (defective), replication competent viruses, which require assistance in order to produce infectious vector

particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids that encode all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR, but that are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsulation. Such helper cell lines include (but are not limited to) Ψ2, PA317 and PA12. A retroviral vector introduced into such cells can be packaged and vector virion produced. The vector virions produced by this method can then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions.

Another targeted delivery system for polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system for use as a delivery vehicle *in vitro* and *in vivo* is a liposome (*i.e.*, an artificial membrane vesicle). It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., *Trends Biochem. Sci.* 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, et al., *Biotechniques* 6:882, 1988).

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity and may be, for example, organ-specific, cell-specific, and/or organelle-specific.

Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticuloendothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

Routes and frequency of administration, as well doses, will vary from patient to patient. In general, the pharmaceutical compositions may be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity or transdermally. Between 1 and 6 doses may be administered daily. A suitable dose is an amount that is sufficient to show improvement in the symptoms of a patient afflicted with a disease associated with NF-κB activation. Such improvement may be detected by monitoring inflammatory responses (*e.g.*, edema, transplant rejection, hypersensitivity) or through an improvement in clinical symptoms associated with the disease. In general, the amount of modulating agent present in a dose, or produced *in situ* by DNA present in a dose, ranges from about 1 μg to about 100 mg per kg of host. Suitable dose sizes will vary with the size of the patient, but will typically range from about 10 mL to about 500 mL for 10-60 kg animal.

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

Identification of an IκB E3 Recognition Motif Using Ubiquitination Assay

This Example illustrates a representative ubiquitination assay, and the use of such an assay to evaluate peptides for the ability to inhibit IκB ubiquitination.

A. *In vitro* Ubiquitination Assay

HA-tagged IκBα or HA-tagged IκBβ cDNAs (Haskill et al., *Cell* *65*:1281-1289, 1991) were translated *in vitro* in wheat germ extract in the presence of ^{35}S-methionine according to the manufacturer's instructions (Promega, Madison, WI). To phosphorylate IκBα or IκBβ, 1 µl of the extract containing the labeled protein was incubated for 90 minutes at 30°C in a reaction mixture having a final volume of 30 µl: 100 µg HeLa or Jurkat cell extract (prepared as described by Alkalay et al., *Proc. Natl. Acad. Sci. USA 92*:10599, 1995), 2mM ATP and 1 µM okadaic acid. During this incubation, the labeled IκB polypeptide was phosphorylated at serines 32 and 36, and associated with the endogenous NF-κB complex (data not shown).

Following incubation, 1 µl of anti-p65 serum was added, and the NF-κB immune complex was immobilized to Protein A-Sepharose® and subjected to *in vitro* ubiquitination in HeLa cell extract as described by Alkalay et al.. Ubiquitinated proteins were separated by SDS-PAGE and visualized by autoradiography.

As shown in Figure 1A, only wild type ^{35}S-pIκBα generated multiply ubiquitinated species (lane 4). Neither ^{35}S-labeled S32/36A mutant of IκBα (lane 1) nor the non-phosphorylated wild type ^{35}S-IκBα (lane 2) were ubiquitinated, and no ubiquitination of pIκBα was seen in the absence of ATP (lane 3).

The physiological relevance of this assay was further documented by comparison of *in vitro* ubiquitination of free ^{35}S-IκB to that of a complex-associated, phosphorylated substrate. Whereas a complex-associated S32/36A mutant was not subject to ubiquitin conjugation in accordance with its *in vivo* fate, free forms of either

mutant or wild type IκBα were readily conjugated (Figure 1B). Similarly, only free, but not a complex-associated lysine 21, 22 mutant of IκBα could be ubiquitinated *in vitro* (data not shown). Thus, while the free IκBα is recognized by the ubiquitin system in a non-discriminatory manner, the complex-associated inhibitor is masked unless it is appropriately phosphorylated.

B. Identification of the IκBα-Ubiquitin Ligase Recognition Motif

To identify the IκBα-ubiquitin ligase recognition motif, various peptides were added at varying concentrations to the reaction mixtures in the presence of the peptidase inhibitor Bestatin (40 μg/ml). The peptides spanned the N-terminal signaling domain of the protein, and were phosphorylated at one or both serine residues (32 and 36), or were unmodified or serine-substituted. These peptides were included in the ubiquitination reaction at different concentrations and tested for inhibition of pIκBα specific ubiquitination. When conjugation of free IκBα was monitored, the translated protein was added directly to the conjugation reaction mixture.

Only peptides that were phosphorylated at both serine 32 and 36 (pIκBα peptides) effectively inhibited pIκBα ubiquitination (Figure 1A, lanes 7, 11-14). A c-Fos phosphopeptide (ppFos, lane 5), a serine 32, 36 to alanine substituted IκBα peptide (p21 S/A, lane 6) and a non-phosphorylated peptide (p21, lane 8) had no detectable effect on the ubiquitination of pIκB at a concentration of 400 μM. The IC_{50} of the phosphorylated IκBα peptides were calculated and representative inhibitory concentrations are shown in Figure 1A. Doubly phosphorylated IκBα peptides inhibited the pIκB conjugation reaction (lanes 7, 11-14) at an IC_{50} of 5 μM. The sequences of these peptides are provided in Table I, above, and in SEQ ID NOs:5-9. In contrast, singly phosphorylated peptides (lanes 9, 10) inhibited the pIκBα conjugation at an IC_{50} of 400 μM. The minimal size peptide tested (pp7, lane 14), merely spanning the signaling phosphorylation site, was sufficient to effectively inhibit the ubiquitination, although at somewhat higher IC_{50} (10 μM). Thus, a peptide comprising residues 21 to 41 of SEQ ID NO:1 comprises a recognition domain for E3 ubiquitin

ligase. Interestingly, lysine residues 21 and 22 are not essential for inhibition, implying that the ubiquitin-system recognition site is distinct from the actual conjugation site.

The specificity of the peptides was tested in two other ubiquitin-conjugation reactions: the conjugation of free wild type (Figure 1B lanes 1-3) or S32/36A mutant IκBα (Figure 1B, lanes 4-6) and the ubiquitin conjugation to the bulk of cellular proteins in HeLa extract (detected by ^{125}I-labeled ubiquitin according to Alkalay et al., Figure 1C). Neither reaction was affected by the addition of an IκBα-ubiquitin ligase recognition motif or a control peptide.

Peptides comprising an IκBα-ubiquitin ligase recognition motif were found to abolish the ubiquitination of the pIκBα related substrate pIκBβ (Figure 1D). Similar to the conjugation of pIκBα, the specific conjugation of the IκBβ also required an associated NF-κB complex (not shown) and prior phosphorylation at the IκBα-homologous residues Ser 19 and 23. An IκBβ substrate prepared in the absence of phosphatase inhibitors was not subject to ubiquitination (Figure 1D, lane 1). Peptides affected pIκBβ ubiquitination at an IC_{50} that was similar to that observed for pIκBα (Figure 1D, lanes 4-7). Hence, it appears that the same enzyme(s) target both IκBs for ubiquitin-dependent degradation.

The inhibitory pIκBα peptides were tested in a complementary ubiquitin-dependent *in vitro* degradation assay (Orian et al., *J. Biol. Chem. 270*:21707, 1995; Stancovski et al., *Mol. Cell. Biol. 15*:7106, 1995). Using this assay, only pIκBα derived from stimulated cells is degraded *in vitro* in a ubiquitin-dependent manner, whereas the non-phosphorylated IκBα from the same cell extract is not subject to degradation. Incorporation of the conjugation-inhibitory phosphopeptides into the degradation assay resulted in stabilization of the pIκBα substrate (Figure 2, lanes 3, 4) whereas the non-phosphorylated peptide agent or a control phospho-Fos peptide had no effect on the specific pIκBα degradation (lanes 5, 6). Trimming the peptides at Lys 21/22 did not diminish the degradation inhibitory effect (lane 4), indicating that the peptides do not abolish pIκBα degradation by exhausting the ubiquitin-proteasome system as conjugatable substrates.

Example 2

Identification of Ubiquitin System Component Involved in Substrate Recognition

This Example illustrates the identification of a specific E3 that is responsible for recognition of pIκB polypeptides.

pIκBα-ubiquitin conjugation and degradation requires a full complement of the ubiquitin system enzymes: E1, a specific E2 derived from the ubiquitin system fraction I, E2F1 (Alkalay et al., *Proc. Natl. Acad. Sci. USA 92*:10599, 1995; Chen et al., *Cell 84*:853, 1996) and a Fraction II-component E3. To identify the ubiquitin system component involved in the substrate recognition, HeLa lysate was fractionated over IκBα phosphopeptide columns, and the flow-through fractions were assayed for pIκBα conjugation. Peptides were coupled to NHS-Sepharose® (Pharmacia) according to the manufacturer's instructions at a concentration of 2 mg/ml. 100 μg of HeLa extract were incubated with 2.5 μl coupled resin in the presence of 0.1% NP40 and 3% ovalbumin for 1 hour at 4°C. The resin was discarded and the unbound material tested in the ubiquitination assay described above.

Whereas a flow-through fraction from a control phosphopeptide column and an S32/36A peptide column retained full IκBα conjugation capacity (Figure 3A, lanes 2, 3) flow-through fractions from two different pIκBα peptides lost their IκBα specific conjugation capacity (lanes 4, 7). The depleted conjugating activity could be complemented by reticulocyte Fraction II (lanes 5, 8) that contains all the known species of E3 enzymes (Ciechanover, *Cell 79*:13, 1994). Complementation could not be obtained by the addition of Fraction I or Fraction I and E1 (lanes 6 and 9, respectively), indicating that the peptide columns depleted an E3 rather than E2 or E1. Again, IκBα lysine residues 21 and 22 were dispensable for retaining the E3 (compare Figure 3A, lane 7 to lane 4), emphasizing the distinction between the substrate recognition and conjugation site. The peptide column depletion was found to be specific for the IκB E3, as all flow-through fractions maintained full activity in random HeLa protein conjugation (as detected by measuring the conjugation of ^{125}I ubiquitin, Figure 3B).

This indicates that a specific E3 is responsible for recognition of the pIκBs at the identified motif.

Example 3

Effect of Representative Peptides on Cellular NF-κB Activation

This Example illustrates the inhibition of cellular NF-κB activation by microinjection of peptides comprising an IκBα-ubiquitin ligase recognition motif.

HeLa cells were plated on a grid coverslips (Cellocate, Eppendorf) 18 hours before microinjection. Microinjection was performed with a 22 amino acid pIκBα peptide (pp21; Table I and SEQ ID NO:9) or a control phospho-Fos peptide (SEQ ID NO:10) using a semi-automated apparatus (Eppendorf). Peptides were injected into the cell cytoplasm at a concentration of 5 mg/ml in 100 mM KCl, 5mM Na_2HPO_4 (pH 7.2), and immediately activated with TNFα (200 units/mL) for either 20 minutes (for NF-κB translocation) or 3 hours (for E-selectin expression). Following activation, the cells were fixed and stained with p65 specific antibodies (Mercurio et al., *Genes & Dev.* 7:705, 1993; Santa Cruz) or monoclonal anti-E-selectin antibodies (R&D Systems).

In the absence of peptide, TNFα induces rapid nuclear translocation of NF-κB into the nucleus, as shown by the p65 nuclear staining of 90% of the cells (*see* Figure 4G, column 2). The pp21 peptide abolished TNFα-stimulated NF-κB activation in 50%-70% of the microinjected cells in several experiments (*see* representative fields in Figures 4A and 4B; and Figure 4G, column 3). In contrast, the control pp-Fos peptide had no effect on the rate of NF-κB induced nuclear translocation, as compared to non-microinjected cells (Figures 4C and 4G, column 4).

To further assess the functional consequences of NF-κB inhibition, the IκB-E3 inhibitory peptide was microinjected into primary human vascular endothelial cells (HUVEC; Chen et al , *J. Immunol 155*:3538, 1995). These cells respond to TNFα stimulation by surface expression of NF-κB regulated adhesion proteins, such as E-selectin. HUVEC cells were plated, microinjected and stimulated as described above.

Three hours post stimulation the cells were fixed and stained for expression of the NF-κB dependent E-selectin. 75%-85% of the HUVEC cells were intensely stained for E-selectin following TNFα stimulation in several experiments. Microinjection of the pp21 peptide resulted in the inhibition of E-selectin expression in 70%-80% of the microinjected cells (Figure 4D; and Figure 4H, column 3). In contrast, the control pp-Fos peptide had no effect on E-selectin expression, as compared to non-microinjected cells (Figures 4F and 4H, column 4). Microinjection of a control, S32/36A substituted IκBα peptide had no effect on the rate of E-selectin expression.

These results demonstrate that the subunit-specific degradation of the signal-induced phosphorylated IκBα and IκBβ is mediated by a specific E3. The recognition domain for E3 ubiquitin ligase is a short sequence, centered around the two signal-acquired phosphoserines conserved in both IκBs, representing the first biologically relevant E3 recognition motif. The specificity in IκB recognition is supported by the context of the phosphorylated substrate: an associated cellular complex masks the substrate from non-specific E3s. This feature restricts the NF-κB inhibitor degradation to the post-stimulation phase, at which it is exposed through site-specific phosphorylation event(s) to the specific ligase. NF-κB activation and its resultant function can be specifically abolished by *in vivo* inhibition of the IκB ligase, using a modulating agent as provided herein.

Example 4

Further Characterization of IκBα Ubiquitination

This Example further illustrates the characterization of the ubiquitin ligase associated with IκBα ubiquitination.

A. Cytokine Stimulation Promotes the Association Between pIκBα and a Specific Ubiquitin-Ligase

To further study the recruitment of components of the ubiquitin machinery by phosphorylated IκBα-complexes, pIκBα/NF-κB complexes were purified

from proteasome inhibited, TNF-α stimulated HeLa cells, and their ubiquitination potential was evaluated. HeLa cells were pre-incubated with the proteasome inhibitor ALLN (150 μM) for 1 hour and stimulated for 10 minutes with TNFα. IκBα/NF-κB complexes were immunoaffinity-purified with goat anti-Rel A (p65) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the cognate p65 peptide (ELFPLIFPAEPAQASGP (SEQ ID NO:21), which was synthetic and purchased from Alfa-Diagnostic, Inc., and then HPLC-purified, analyzed by mass spectrometry, verified for the predicted structure and proven to be over 85% pure).

The immunopurified fraction was supplemented with various components of the ubiquitin system and subjected to *in vitro* ubiquitination. In particular, the fraction was supplemented with 0.2 μg purified El and 1 μg purified recombinant UBC5C (Jensen et al., *J. Biol. Chem. 270*:30408-30414, 1995) and incubated for 90 minutes at 37°C in reaction buffer containing: 50mM Tris (pH 7.6), 2mM $MgCl_2$, 1mM DTT, 20nM okadaic acid, 1mg/ml bovine ubiquitin (Sigma) and 5mM ATPγS (Sigma). The reaction mix was then boiled in SDS-buffer and the sample analyzed by SDS-PAGE (8.5%) and phospho-imaging.

The addition of ubiquitin, purified E1 and a specific E2, UBC5C, was found to be sufficient to generate the full capacity IκBα-ubiquitin conjugating activity (Figure 5, lane 2), evident in the accumulation of high-molecular mass species that reacted with IκBα specific antisera. This activity was El-dependent (compare lanes 1 and 2), and was not provided by the corresponding immunopurified fraction from non-stimulated HeLa cells (compare lanes 4, 5, 6). As the stimulated HeLa fraction contained both phosphorylated and non-phosphorylated IκBα, the observed conjugates could be derived from either IκB species.

To determine the source of the IκBα-conjugates, the ubiquitination reactions were performed in the presence of a pIκBα peptide (pp12; CDRHDS[PO3]GLDS[PO3]; SEQ ID NO:22) (lane 7) or a serine/glutamic-acid substituted IκBα peptide (p12S/E) (lane 8). Both peptides were synthetic, purchased from Alfa-Diagnostic, Inc., and then HPLC-purified, analyzed by mass spectrometry, verified for the predicted structure and proven to be over 85% pure. IκBα peptides

were added at the indicated concentrations to the reaction mixtures in the presence of the peptidase inhibitor Bestatin (40 μg/ml). Only pp12 abolished the formation of polyubiquitin-IκBα conjugates, indicating that ubiquitination was specific for pIκBα (Yaron et al., *EMBO J. 16*:6486-6494, 1997).

B. Phosphorylation is Necessary and Sufficient to Recruit Specific Ubiquitin-Ligase Activity

The finding that E1 and E2 specifically complemented pIκBα-conjugation of the stimulated HeLa fraction, but failed to complement a non-stimulated fraction, could be explained in several ways: a) HeLa stimulation activates a specific pIκB-ubiquitin ligase, b) HeLa stimulation modifies the substrate, thus rendering it liable to ubiquitination, or c) HeLa stimulation is necessary for modifying both the substrate and the ligase. To distinguish among these possibilities, a recombinant, constitutively active IKK2 protein (IKK2-EE) was used (Mercurio et al., *Science 278*:860-66, 1997). This protein phosphorylates IκBα at serine 32/36 similarly to a TNFα activated IKK-complex.

Following immunoprecipitation of ^{35}S-labeled IκBα/NF-κB complexes from a non-stimulated HeLa lysate previously incubated with recombinant ^{35}S-labeled IκBα, the complexes were phosphorylated by the recombinant IKK2-EE, eluted with the p65 cognate peptide and subjected to *in vitro* ubiquitination. After incubation with IKK2-EE, nearly all of the ^{35}S-IκB was phosphorylated. Yet, the addition of ubiquitin, E1 and UBC5C did not result in pIκBα phosphorylation (Figure 6, lane 2). Therefore, IκB phosphorylation by IKK was not sufficient to promote its ubiquitination in the presence of E1 and E2. Conceivably, pIκBα ubiquitination requires an additional component of the HeLa lysate that was not co-immunopurified from non-stimulated cells.

To confirm this hypothesis, immuno-bound IκBα/NF-κB complexes were incubated with a non-stimulated HeLa lysate, either directly or following IKK2-EE phosphorylation, washed extensively with high-salt buffer and eluted with the p65 peptide. Indeed, incubation of the phosphorylated IκB complexes (Figure 6 lane 3), but

not of the non-phosphorylated ones (lane 1), with the HeLa lysate, provided the pIκB-ligase component(s) necessary for pIκBα conjugation. No signal was obtained when E1 or E2 were omitted from the reaction, confirming that the signal at the top of the gel represents poly-ubiquitin IκBα-conjugates (lanes 5, 6). TNFα stimulated HeLa-lysate was not superior over a non-stimulated lysate in providing the necessary ligase component.

The inhibitory effect of pp12 on pIκBα-ubiquitination (Figure 5) suggested that the essential HeLa component associates specifically and stably with the pIκBα recognition motif during the incubation period and later functions in pIκB-ubiquitin conjugation. To test this assumption, we included in the incubation step pp12 or the control peptide p12S/E, which was removed together with the HeLa lysate, before eluting the fractions. The addition of pp12 (Figure 6, lane 4), but not of p12S/E (lane 5), abrogated the ubiquitin-ligase activity associated with the pIκB-complex, while preserving the integrity of the substrate. This was evident in the ability of the peptide-treated fractions to undergo ubiquitination in the presence of Reticulocyte Fraction II as an E3 source (Alkalay et al., *Mol. Cell Biol. 15*:1294-301, 1995). Several conclusions may be drawn from this experiment:

1) A ubiquitin-ligase component essential to pIκBα ubiquitination is recruited by the IκBα/NF-κB complex from the HeLa lysate following IKK phosphorylation.

2) This conjugation-promoting component is contained in a non-stimulated HeLa lysate, indicating that there is no need to activate the ubiquitin-ligase by TNF-stimulation.

3) The essential ligase component is apparently specific and associates with IκB through a direct interaction with the pIκB recognition motif (proved by pp12 inhibition of pIκBα-conjugation).

C. Isolation of the Specific Ubiquitin-Ligase Component that Recognizes pIκBα

HeLa extract (250mg) was incubated with 250 µl anti-p65 immunobeads. Following four washes in buffer A (1M KCl, 0.5 % NP40, 50mM Tris

buffer pH 7.6, 1mM DTT) and one wash in buffer B (50mM Tris buffer, pH 7.6, 1mM DTT), half the beads were subject to *in vitro* phosphorylation with IKK and half underwent mock-phosphorylation. The beads were washed twice in buffer A and once in buffer B, agitated with 100mg HeLa extract in the presence of 1 μm okadaic acid for 30 min at 25°C, washed four times with buffer A, once in buffer A and eluted with 1mg/ml p65 peptide. A similar experiment was performed with 10 mg ^{35}S-metabolically-labeled HeLa cell lysate (100 μCi/ml Met/Cys for 8 hours) and 25 μl p65-immunobeads. Eluate-fractions derived from both the hot and cold lysates were mixed, boiled in SDS-sample buffer and analyzed by 7.5% SDS-PAGE and autoradiography. Gel slices corresponding to the autoradiogram signals were excised and their protein-bands sequenced by mass-spectrometry, as described below.

Three immunoaffinity-purified fractions were compared by SDS-page analysis (Figure 7A): 1) a fraction containing IκBα/NF-κB complexes that was not phosphorylated by IKK2-EE, but incubated with HeLa lysate; 2) a fraction subjected to IKK2-EE phosphorylation and subsequent incubation with HeLa lysate; 3) a fraction phosphorylated by IKK2-EE, but not incubated with HeLa lysate. All incubations were performed on immunobead-immobilized complexes, which were then extensively washed and eluted with the p65 peptide.

SDS-PAGE analysis of the three fractions revealed pattern-changes due to IKK phosphorylation or to further immuno-adsorption of IκBα/NF-κB proteins, but did not discern any protein recruited to the IκB-complex following IKK phosphorylation. The complexity of the protein staining could obscure the presence of any recruited protein migrating along with an immunopurified protein. To identify the recruited protein, mass-spectrometry analysis was performed on a dozen Colloidal Blue-stained bands derived from fractions 1 and 2. This analysis revealed the presence of nearly the full spectrum of the Rel family proteins and IκBα: NF-κB1 (p105), NF-κB2 (p100), RelA (p65), p50, p49, C-Rel, IκBα and IκBε. Only a few other proteins were co-immunoprecipitated with the IκB/NF-κB complex, particularly GRP78/Bip, Hsp 70 and Hsc 70.

To circumvent the possible masking of the putative pIκB-ubiquitin ligase, we replaced the ligase source with ^{35}S-biosynthetically-labeled HeLa lysate and traced the IκBα-associated proteins by SDS-PAGE analysis and autoradiography (Figure 7B). In parallel, the various fractions were tested for their ubiquitin-ligase capacity. The band-pattern of the active fraction (lane 2) was compared with that of the non-active one (lane 1). Four ^{35}S-protein bands with a molecular mass of 54, 58, 61 and 64 kD were distinguished in lane 2. Some of these protein bands could represent components of the ubiquitin ligase that recognizes pIκBα directly whereas others might have associated with pIκBα indirectly or with another component of the IKK-phosphorylated complex. To sort out the ligase component that recognizes pIκBα directly, pp12 or the control peptide p12S/E was added to the radiolabeled HeLa lysate, which was then incubated with the immuno-bound IκBα/NF-κB complex. A comparison of the eluted fractions showed that of the four distinctive bands present only in fraction 2, three bands were eliminated by the specific pp12 peptide (p54, p58 and p61), whereas only the 64 kD band persisted in the presence of pp12 (Figure 7B, compare lanes 2 and 3). The control peptide did not affect the association of any of the distinctive proteins with pIκBα (lane 4). Two of the pIκBα interacting proteins, p58 and p54, were consistently present and always associated with the specific ubiquitin-ligase activity.

Example 5

Identification of Human E3 Ubiquitin Ligase

This Example illustrates the isolation and characterization of human E3 ubiquitin ligase.

The 54 and 58 kD bands described in the previous Example were excised from a ligase-positive and a ligase-negative (HeLa lysate incubated with a non-phosphorylated IκBα-complex) lane, the proteins digested *in situ* (Shevchenko et al., *Anal. Chem. 68*:850-858, 1996) and the tryptic peptides thus obtained were sequenced by nanoelectrospray mass spectrometry (Wilm et al., *Nature 379*:466-469, 1996).

Protein bands were reduced in-gel, S-alkylated and digested in-gel with an excess of trypsin (overnight at room temperature) as described (Shevchenko et al., *Anal. Chem. 68*:850-858, 1996; Wilm et al., *Nature 379*:466-469, 1996). Pieces of gel were extracted and the resulting peptide mixtures were concentrated and desalted, using a micro-column containing 50 nl of Poros R2 material (Perceptive Biosystems, Framingham, MA). Peptides were eluted with 1 μl of 60% methanol, 5% formic acid directly into a nanoelectrospray needle. Nanoelectrospray spectra were recorded on a quadrupole time-of-flight mass spectrometer (QqTOF, Perkin-Elmer Sciex, Toronto, Canada). Peptide sequence tags (Mann and Wilm, *Anal. Chem. 66*:4390-4399, 1994) were assembled from fragmentation spectra and searched against a non redundant protein sequence database (nrdb) maintained at the European BioInformatics Institute (EBI, Hinxton Park, England) using the program PeptideSearch (Mann and Wilm, *Anal. Chem. 66*:4390-4399, 1994).

Mass spectra of the 54 kD gel band revealed a complex peptide mixture (Figure 8A) from which several peptides were selected for fragmentation. Proteins identified by peptide sequence tag searching (Mann and Wilm, *Anal. Chem. 66*:4390-4399, 1994) included NF-κB1 (p50), IκB kinase α, IκBε, RelB, tubulin beta-1 chain, and thyroid receptor initiator binding protein. To identify the protein associated with the E3 activity, additional peptides, present in small amounts, were selected for sequencing by comparing the spectrum of the 54 kD bands from the active fraction with that of a similar band from the non-active one (Figure 8B). The peptide sequence tag (1587.81) VVNV (SEQ ID NO:23) (1999.09) was derived from the fragmentation spectrum shown in Figure 8C and unambiguously identified as AAVNVVDFDDKYIVSAS (SEQ ID NO:24). Further spectra identified the peptides LEGHEELVR (SEQ ID NO:25), LVVSGSSDNTIR (SEQ ID NO:26), IQDIETIESNWR (SEQ ID NO:27) and VISEGMLWK (SEQ ID NO:28). The first four fragments have sequences present within the human F-box/WD protein β-TrCP (Margottin et al., *Mol. Cell 1*:565-574, 1998). However, the fifth peptide (VISEGMLWK (SEQ ID NO:28)) matches that of a peptide from the Drosophila Slimb protein (*see* Jiang and Struhl, *Nature 391*:493-496, 1998), which is highly homologous

to human β-TrCP. Further sequencing identified the human E3 ubiquitin ligase nucleotide sequence provided in Figure 9 (SEQ ID NO:15), and the predicted protein sequence provided in Figure 10 (SEQ ID NO:16). Thus, the human E3 ubiquitin ligase appears to be a novel member of the β-TrCP/Slimb family of homologous proteins.

Example 6

Further Characterization of E3 Ubiquitin Ligase Activity

This Example further illustrates the ubiquitin ligase activity of the human E3 ubiquitin ligase family members β-TrCP and Slimb.

The ability of these proteins to bind pIκBα specifically and assist in its ubiquitination was examined in a cell-free system. The IκBα/NF-κB complex was immunopurified from HeLa cells and the immune complex was either phosphorylated with IKK2-EE or mock-phosphorylated as described above. It was then incubated with the following immobilized FLAG-tagged E3 family members immunoprecipitated from transfected 293 cells: mouse β-TrCP (mβ-TrCP), human β-TrCP (hβ-TrCP), human β-TrCP with a deletion of the F box region residues 122-168 (Δβ-TrCP) and the Drosophila Slimb protein. The bound material was analyzed by Western blotting with anti-IκBα and anti-FLAG antibodies. All of these proteins exclusively bound IKK-phosphorylated, but not mock-phosphorylated, IκBα (*see* Figure 11A). However, the human and mouse β-TrCP bound IκBα far better than the highly homologous Drosophila protein (compare lanes 2, 4, 6 and 8). Δβ-TrCP bound pIκBα even better than the wild type protein, indicating that the F-box region was dispensable for binding. Furthermore, β-TrCP binding was abrogated by a peptide representing the pIκBα recognition motif (pp10; DRHDS(PO_3)GLDS(PO_3)M (SEQ ID NO:29); *see* Figure 11B, lane 3), but not by the control peptide (lane 4), specifying the site of pIκBα recognition of the conserved DS(PO_3)GLDS(PO_3) (SEQ ID NO:30) sequence.

To evaluate the effect of binding on ubiquitination, the E3 family members and the deletion mutant were used as a source of E3 activity in pIκBα ubiquitination. In the presence of E1 and E2 (UBC5C), the wild type β-TrCP proteins

facilitated the ubiquitination of pIκBα, but not of the non-phosphorylated IκBα (*see* Figure 11C, lanes 1-4). Δβ-TrCP, devoid of the F-box protein-protein interaction module, failed to promote ubiquitination (lanes 7 and 8), in spite of its binding capacity (Figure 11A, lane 6). Although Slimb facilitated some pIκBα ubiquitination, it was at least ten-fold less efficient than the human and mouse β-TrCP (based on similar FLAG-tag expression levels), corresponding to its weaker activity.

The modular design of these family members and the *in vitro* analysis described herein suggested that deletion of the F-box would result in a protein that functions as a dominant negative molecule *in vivo*. In fact, transient over-expression of the Δβ-TrCP inhibited the degradation of endogenous IκBα in stimulated Jurkat cells, resulting in accumulation of pIκBα (Figure 12A). Consequently, activation of NF-κB was inhibited (Figure 12B). NF-κB activation was specific, as Δβ-TrCP did not affect activation of an NF-AT reporter. Of note is the fact that NF-κB inhibition was also observed with wild type Slimb, whereas the expression of wild type human β-TrCP was not inhibitory (Figure 12B). Therefore, overexpression of wild type Slimb has a dominant negative effect on NF-κB activation, probably linked to its relatively poor pIκBα ubiquitination activity (Figure 11B).

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SUMMARY OF SEQUENCE LISTING

SEQ ID NO:1 is amino acid sequence of IκBα

SEQ ID NO:2 is DNA sequence of IκBα

SEQ ID NO:3 is amino acid sequence of IκBβ

SEQ ID NO:4 is DNA sequence of IκBβ

SEQ ID NO:5 is amino acid sequence of pp7

SEQ ID NO:6 is amino acid sequence of pp11

SEQ ID NO:7 is amino acid sequence of pp15

SEQ ID NO:8 is amino acid sequence of pp19

SEQ ID NO:9 is amino acid sequence of pp21

SEQ ID NO:10 is amino acid sequence of phospho-Fos peptide

SEQ ID NO:11 is amino acid sequence of pp21 S/A

SEQ ID NO:12 is amino acid sequence of HA-tagged IκBα

SEQ ID NO:13 is amino acid sequence of HA-tagged S32, 36 IκBα

SEQ ID NO:14 is amino acid sequence of HA-tagged IκBβ

SEQ ID NO:15 is DNA sequence of human E3 ubiquitin ligase

SEQ ID NO:16 is predicted amino acid sequence of human E3 ubiquitin ligase

SEQ ID NO:17 is DNA sequence of human β-TrCP

SEQ ID NO:18 is amino acid sequence of human E3 β-TrCP

SEQ ID NO:19 is phosphorylation site of IκBα

SEQ ID NO:20 is retrieved β-TrCP sequence

SEQ ID NO:21 is amino acid sequence of cognate p64 peptide

SEQ ID NO:22 is amino acid sequence of pIκBα peptide pp12

SEQ ID NO:23 is peptide sequence tag of human E3 ubiquitin ligase

SEQ ID NO:24 is peptide from human E3 ubiquitin ligase

SEQ ID NO:25 is peptide from human E3 ubiquitin ligase

SEQ ID NO:26 is peptide from human E3 ubiquitin ligase

SEQ ID NO:27 is peptide from human E3 ubiquitin ligase

SEQ ID NO:28 is peptide from human E3 ubiquitin ligase

SEQ ID NO:29 is amino acid sequence of pIκBα recognition motif

SEQ ID NO:30 is conserved pIκBα sequence